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An improved, high-throughput method for detection of bluetongue virus RNA in *Culicoides* midges utilizing infrared-dye-labeled primers for reverse transcriptase PCR

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Abstract

A new rapid (less than 6 h from insect-to-results) high-throughput assay that is sensitive and specific for detecting BTV RNA in *Culicoides* biting midges is reported. Homogenization and extraction of nucleic acids from individual *Culicoides* specimens were performed in a 96-well plate format using specialized beads in a homogenization buffer compatible with cell culture and RNA extraction. A portion of homogenate (10%) from each specimen was retained for confirmatory infectious virus isolation, while the remaining 90% was used for RNA extraction. The RNA was used in a single step reverse transcriptase PCR (RT-PCR) reaction with infrared (IR)-dye-labeled primers. The RT-PCR products were visualized in agarose gels with an infrared scanner. The adaptation of IR-dye-labeled primers in combination with a one step RT-PCR resulted in a detection limit of 0.5 pfu of purified BTV RNA. All 24 serotypes of BTV prototype strains and none of the 8 serotypes of the closely related epizootic hemorrhagic disease virus (EHDV) prototype strains were detected.
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Keywords: Bluetongue virus; Infrared-dye; Reverse transcriptase PCR; *Culicoides sonorensis*

1. Introduction

Bluetongue virus (BTV) is the prototype *Orbivirus* within the *Reoviridae* family. The double stranded, segmented RNA arbovirus is vectored by several *Culicoides* spp. biting midges. BTV and *Culicoides* vectors exist in subtropical, tropical and temperate regions throughout the world. Changes in climatic conditions have allowed the spread of exotic strains of BTV into Europe, Asia, and North America (Gibbs and Greiner, 1994; Mellor and Wittmann, 2002; Purse et al., 2005; Tabachnick et al., 1996). BTV is on the Office International des Epizooties (OIE) list of notifiable diseases because of the severe socio-economic impacts caused by BTV outbreaks. BTV vectors have been identified as the critical component in the spread of the virus and the changing global epidemiology of BTV. OIE therefore emphasizes the need for surveillance of competent *Culicoides* vectors in areas at high risk for expanded habitation based on historical, geographic and climatic factors (MacLachlan and Osburn, 2006; OIE, 2004). The recent OIE recommendations necessitate the development of effective, reliable, high-throughput methods to survey competent *Culicoides* and potential vectors for all BTV serotypes.

Current protocols for BTV detection in biting midges involve time consuming steps for sample (single insect) homogenization, preparation, and assay (Wieser-Schimpf et al., 1993). For example cell culture techniques for BTV identification may not rapidly detect all BTV particles within a sample. Standard RT-PCR and nested PCR assays for BTV RNA require multiple steps for reverse transcriptase reaction, and primary and/or secondary PCR amplification (Akita et al., 1992; Aradaib et al., 1998, 2003; Dangler et al., 1990a; Katz et al., 1993; Wilson and Chase, 1993). Finally, real time PCR protocols do not detect all 24 serotypes (Jimenez-Clavero et al., 2006; Orru et al., 2004; Wilson et al., 2004).

Initial PCR detection assays for BTV involved either standard or single amplification reverse transcriptase PCR (RT-PCR) and have detection sensitivities of 1 pg to 17 fg BTV RNA (Akita et al., 1992; Dangler et al., 1990b; McColl and Gould, 1991; Wade-

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Evans et al., 1990). The incorporation of a second amplification step, and the development of nested PCR protocols, increased detection sensitivities to 0.1 fg and one cell culture infective dose ($CCID_{50}$)/ml. However, nested PCR often involves five to seven product manipulations that increase the possibility of cross-contamination and false positives (Aradaib et al., 1998; Katz et al., 1993; Parsonson and McColl, 1995; Shad et al., 1997; Wilson, 1999; Wilson and Chase, 1993). Real time PCR for arbovirus detection is gaining popularity (Mackay et al., 2002). BTV detection by real time PCR is efficient and can be very sensitive (Jimenez-Clavero et al., 2006; Orru et al., 2004; Wilson et al., 2004). Jimenez-Clavero et al. (2006) recently reported high-throughput real time PCR detection of BTV in blood samples with detection sensitivities equivalent to nested PCR. The assay targets BTV segment 5 (NS1 protein) which is involved in virus replication. In the study, 9 of 24 reference strain serotypes could not be detected (serotypes 4, 7, 10, 13, 16, 19, 20, 21, and 24), because of nucleotide sequence variations in the regions of probe and the 5′-primer binding site.

Increasing the sensitivity of a standard RT-PCR assay eliminates the need to identify additional primer or probe binding sites. IR-dye-labeled primers have been used in place of radioisotope labeled primers for various applications where enhanced sensitivity is needed. Applications include electrophoretic mobility shift (EMS) assays, amplified fragment length polymorphism (AFLP) analysis, single nucleotide polymorphism (SNP) mapping, DNA sequencing, short tandem repeat (STR) analysis, and DNA footprinting (Ford et al., 2000; Machida et al., 1997; Middendorf et al., 1992; Myburg et al., 2001; Ricci et al., 2003; Somboonthum et al., 2005). The spectral characteristics of these dyes allow for detection in the near IR (700–800 nm) range. At this spectral range, background fluorescence is low, therefore, detection of minute amounts of product can be differentiated from background (Middendorf et al., 1992). Primer extension and DNA sequencing applications previously described with IR dye labeled primers indicate that the dye conjugates do not interfere with reverse transcriptase and DNA polymerase activity, while retaining a high level of selectivity and sensitivity (Ricci et al., 2003; Somboonthum et al., 2005). In coupling this highly sensitive dye with a one step RT-PCR assay, a sensitive assay for pathogen detection was developed. This infrared reverse transcriptase PCR (IR-RT-PCR) assay is suitable for high-throughput analysis of individual *Culicoides* for BTV. The IR-RT-PCR assay is sensitive, rapid, economical, reduces false positives (due to cross-contamination), and is specific to all 24 BTV serotypes. Method development also focused on efficiency and economy of sample preparation so that the processing time and effort was decreased and the limited sample volume could easily and effectively be used in both cell culture and IR-RT-PCR assays.

2. Materials and methods

2.1. Virus

BTV serotype 11 (station strain; 6.8 × 106 plaque forming units (pfu)/ml), was used in all assays for IR-RT-PCR sensitivity, cell culture detection, and oral infection of *Culicoides*. Double stranded RNA from all 24 serotypes of BTV (prototypes) and all 8 epizootic hemorrhagic disease virus (EHDV) serotypes were obtained from the Arthropod-Borne Animal Diseases Research Laboratory (ABADRL) collection (USDA, ARS, ABADRL, Laramie, WY) and were used for determining primer specificity.

2.2. IR-RT-PCR assay sensitivity

BTV serotype 11 RNA was isolated from stock cell culture (400 μl) containing 6.8×10^6 pfu/ml with the Mag Attract Virus Mini M48 Kit and a BioRobot M48 (Qiagen, Inc., Valencia, CA, USA). The extraction was performed as specified by the manufacturer and eluted in a final volume of 50 μl.

IR-RT-PCR assay sensitivity tests were performed by the analysis of three different dilution sets each carried out in triplicate. Set 1: 500, 50, 5, 0.5, and 0.05 pfu/reaction. Set 2: 250, 25, 2.5, 0.25, and 0.025 pfu/reaction. Set 3: 100, 10, 1, 0.1, and 0.01 pfu/reaction. A non-template control was included in each replicate.

BTV specific primers BTV 12F (5′-TCGCTGCCATGCTATCCG-3′) and BTV 246R (5′-CGTACGATGCGAATGCAG-3′) (Akita et al., 1992) were synthesized with a 5′ IR-Dye 800 chromophore (LI-COR, Inc., Lincoln, NE). These primers are specific to the highly conserved regions of the S10 gene (nonstructural protein 3), located at nucleotide positions 12 and 246, for forward and reverse primers, respectively (251 bp product). Five hundred nanomoles of each of the IR-dye-labeled primers was added (1 μl) to each well of a 96-well thin walled PCR plate (Applied Biosystems, Foster City, CA). Each nucleic acid sample (5 μl) was then added to the PCR plate. The RNA/primer mixture was heat denatured at 95 °C for 3 min then quickly quenched in an ethanol ice bath for at least 5 min before a 1 min centrifugation at 2000 × *g* at 4 °C. The plate was placed on ice and the RT-PCR reagent mix (1× Taqman One-Step RT-PCR master mix and multiscribe) was added to each well. Thermal cycler conditions (GeneAmp 9600, Applied Biosystems) were as follows: 48 °C for 30 min, 95 °C for 10 min, 40 PCR cycles of 95 °C for 15 s and 60 °C for 1 min, followed by 4 °C hold.

All 96 RT-PCR products were run with 1.4× orange loading dye (LI-COR Inc.) with an impact, 8-channel pipette (Matrix Technologies Corp., Hudson, NH, USA) on a 3% agarose gel (Owl Separation Systems, Inc., Portsmouth, NH, USA) at 100 V for 45 min. The gels were loaded quickly, and the gel box was covered with aluminum foil to reduce chromophore exposure to light. Bands were visualized by scanning with an Odyssey (LI-COR) instrument with the 800 channel at medium sensitivity.

2.3. IR-RT-PCR assay specificity

Assay specificity tests were preformed as described above (Section 2.2), except using purified dsRNA obtained from the ABADRL BTV reference collection. Samples included 1 ng each of the 24 reference strains of BTV and the 8 known reference strains of EHDV. These assays were preformed in triplicate and included a non-template control for each replicate.

2.4. High-throughput processing of individual Culicoides for BTV IR-RT-PCR and cell culture detection sensitivities

Individual *Culicoides sonorensis* (Ausman lineage reared at the ABADRL; Campbell et al., 2004) were placed into the wells of a chilled 96 sample collection microtube rack with a single, surface sterilized, 3.96 mm gold plated tungsten bead (Spirit River Inc., Roseburg, OR, USA) and 110 μl homogenization buffer (20% fetal bovine serum, 50 μg/ml streptomycin, 50 U/ml penicillin, and 2.5 μg/ml amphotericin B in phosphate-buffered saline; Shi et al., 2001).

Three sets of BTV-11 stock culture dilutions were prepared with homogenization buffer and added to the tubes (above) so that each dilution set was tested with four replicates at the following concentrations: 5000, 1000, 500, 100, 50, 10 and 0 pfu/well (a final well was left empty for each dilution set and used for the non-template/cell culture control). Samples were homogenized by mechanical agitation in a Tissue Lyser^{TM} (Qiagen, Inc., Valencia, CA) twice for 1 min at 25 rotations/s. The rack was flipped between the two agitations as recommended by the manufacturer. Homogenates were then centrifuged for 2 min at 2000 × *g* at room temperature. An aliquot (11 μl) of the homogenate was removed from each sample and placed in a duplicate plate with 39 μl of gnat antibiotic solution (M199E with 10% fetal bovine serum, 400 μg/ml streptomycin, 400 U/ml penicillin, 200 μg/ml gentamycin, 25 μg/ml ciprofloxacin and 5 μg/ml fungizone) in each well for cell culture. The remainder of the homogenate was used for RNA isolation.

Virus detection in cell culture was performed according to Mecham (1993), with the following modifications. The homogenate was incubated with gnat antibiotic medium for 2–3 days at 4 °C before plating to assure that the homogenate was free of fungal or bacterial contaminants. The homogenate was plated in duplicate 96 well plates (25 μl/well) with 100 μl VERO cells/well (3.5×10^5 cells/ml), and incubated for 7 days at 37 °C with 5% CO_2. A positive was indicated when one or both of the duplicates exhibited a cytopathic effect (CPE).

High-throughput nucleic acid isolation was performed using an RNeasy 96 Kit (Qiagen, Inc.) as per the manufacturer's instructions without DNase I treatment and the following modifications (per well). RLT buffer (350 μl, prepared with 0.1% β-mercaptoethanol) with 5.7 ng/μl carrier RNA (Qiagen, Inc.) was added to 100 μl homogenate. All samples were mixed by pipetting. Absolute ethanol (250 μl) was added to each of the samples and mixed before applying to the RNeasy 96 plate. The columns were washed twice with 1 ml RPE buffer, and once with 0.5 ml 80% ethanol. All wash steps were performed on the QIAvac 96 Vacuum Manifold (Qiagen, Inc.), and the subsequent column drying and elution steps were performed as described by Qiagen spin technology with the exception that microtiter plates (Greiner Bio-One, Inc., Longwood, FL) were used to support the RNeasy plate during drying and elution by centrifugation in an Alegra centrifuge (Beckman Coulter, Fullerton, CA), at 2000 × *g* for 2 min. Eluates were stored at −20 °C until used for BTV detection by IR-RT-PCR.

Five microliters of each BTV eluate of *Culicoides* spiked with BTV-11 dilutions was assayed as above. Samples reflect approximately 5% of the original spike of BTV. Each replicate included the following calculated BTV amounts: 250, 50, 25, 5, 2.5, 0.5, and 0 pfu/reaction. A non-template control was also included for each replicate.

2.5. Experimental infection of Culicoides

Five batches of *Culicoides* were challenged with virus blood meals. Three-day-old adult female *Culicoides sonorensis*, Ausman lineage (ABADRL) were fed infectious blood meals for 2 h using an artificial feeder (Hunt and McKinnon, 1990). The virus blood meal consisted of 1 ml 6.8×10^6 pfu/ml BTV-11 mixed with an equal volume of defribrinated sheep blood. Engorged females were sorted into cages, given 10% sucrose water ad libitum and held at 24 °C for 10 days (extrinsic incubation period and to assure that all remnants of undigested virus blood meal had been excreted) (Hunt and McKinnon, 1990; Hunt et al., 1989; Sieburth et al., 1991). At 10 days, surviving insects were stored at −80 °C until assayed. Positive controls (5–6 per feeding) were fed as described above, and then frozen immediately after the 2 h feeding. These insects were processed and assayed by both cell culture and IR-RT-PCR as described previously (2.4).

3. Results

3.1. IR-RT-PCR assay sensitivity and specificity

IR-RT-PCR assay sensitivity was determined by testing three sets of serial dilutions of purified BTV RNA in triplicate (Fig. 1). The serial RNA dilutions showed a consistent limit of detection of BTV RNA at 0.5 pfu (set A). RNA was also detected at 0.25 pfu in two of three replicates (set B).

Specificity and cross-reactivity was assessed by screening the 24 prototype strains of BTV and 8 prototype strains of EHDV. All 24 BTV prototype serotypes were detectable by IR-RT-PCR, with no cross-reactive products observed for EHDV prototype samples (Fig. 2).

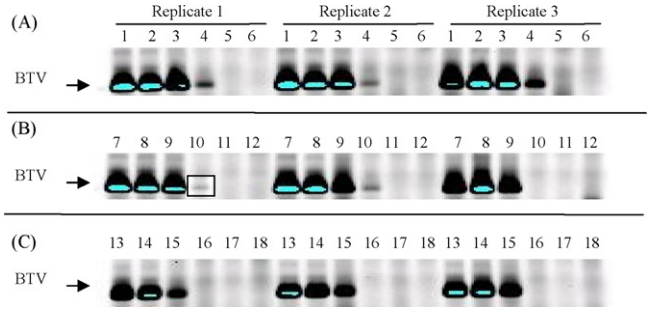

Fig. 1. IR-RT-PCR assay sensitivity. A series of three sets of BTV-11 serial dilutions were assayed for the presence of BTV by IR-RT-PCR. Each set of dilutions were performed in triplicate with the following concentrations: Set A, 1–5 (500, 50, 5, 0.5, and 0.05 pfu/reaction, respectively); Set B, 7–11 (250, 25, 2.5, 0.25, and 0.025 pfu/reaction, respectively); and Set C, 13–17 (100, 10, 1, 0.1, and 0.01 pfu/reaction, respectively). Numbers 6, 12, and 18 are non-template controls, light blue areas within bands represent high intensity signals, and the boxed band indicates an increase in exposure.

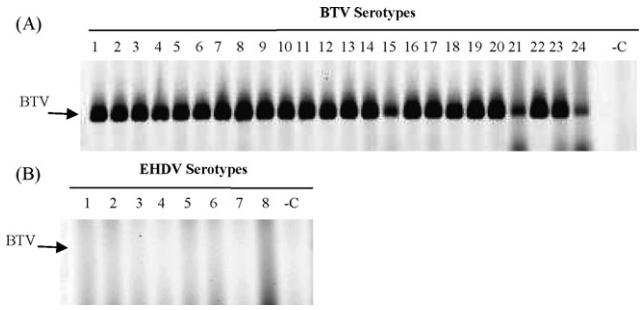

Fig. 2. IR-RT-PCR assay specificity. Detection of BTV in (A) all reference strains of the 24 BTV serotypes (BTV 1–24) and (B) the 8 reference strains of the 8 known EHDV serotypes (EHDV 1–8). Non-template control is designated -C.

3.2. High-throughput sample preparation and IR-RT-PCR

To determine the sensitivity of IR-RT-PCR with high-throughput processed *Culicoides* samples, four replicates of three BTV-11 dilutions were prepared and added to single *Culicoides* midges (with bead and homogenization buffer) in a 96 well format. A portion (10%) of each homogenate was removed and used for cell culture detection of virus, while the remainder of each sample was used for RNA isolation and IR-RT-PCR assay.

Culicoides homogenization was complete after 2×1 min cycles on the Tissue Lyser^{TM}, as assessed by visual inspection. High-throughput processing of individual *Culicoides* spiked with dilutions of BTV showed detection sensitivity of 0.5 pfu by IR-RT-PCR.

Cell culture assaying of the samples resulted in CPE in all replicate wells containing 25 pfu, but CPE in only one of four replicates at 5 and 2.5 pfu/well. No CPE was observed in wells with 0 pfu (*Culicoides* negative control), nor the cell culture negative controls. All dilutions and replicates of 250, 50, 25, 5, 2.5, and 0.5 pfu/well (initial inoculation of 5000, 1000, 500, 100, 50, and 10 pfu/insect, respectively) were positive when evaluated by IR-RT-PCR (Fig. 3).

3.3. BTV detection in experimentally infected Culicoides

A total of 232 *Culicoides* were assayed by both cell culture and IR-RT-PCR, including 33 fed a non-viremic blood meal, 26 virus challenged flies frozen immediately after feeding (positive controls), and 173 flies fed a viremic meal (challenged) and held for 10 days. At 10 days post-feeding (dpf) 82.1% of the challenged specimens assayed by IR-RT-PCR were positive for BTV RNA. When the same specimens were assayed by cell culture, approximately 18% showed signs of CPE. All unchallenged flies were negative by both assays. The IR-RT-PCR assay detected BTV RNA in all positive controls, while cell culture identified 88.5% as having CPE (Table 1).

Table 1
BTV detection of experimentally infected *Culicoides* by cell culture and IR-RT-PCR

Culicoides treatment	Cell culture[a]	IR-RT-PCR[a]
Unchallenged	0/33 (0%)	0/33 (0%)
Challenged, 0 dpf	23/26 (88.5%)	26/26 (100%)
Challenged 10 dpf	31/173 (17.9%)	142/173 (82.1%)

[a] No. positive/no. challenged, percent positive in parentheses.

4. Discussion

Assay detection limits were determined to be 0.5 pfu/reaction, i.e., 100% for all three replicates. At 0.25 pfu/reaction, the IR-RT-PCR assay detected the presence of BTV in two of the three replicates. These sensitivities are comparable to nested PCR and real time PCR assays which are currently being used in OIE protocols for BTV detection and the testing of cattle for BTV in Spain, respectively (Jimenez-Clavero et al., 2006; OIE, 2004).

Unlike the nested PCR (OIE, 2004) real time PCR (Jimenez-Clavero et al., 2006) and IR-RT-PCR assays are faster and simpler to perform. In both the IR-RT-PCR and real time PCR methods there are two manipulation steps versus seven steps for the OIE nested PCR procedure. Efficiency is increased with less manipulation of the sample and the possibility of cross-contamination and false positives is reduced.

Specificity of the IR-RT-PCR assay includes all 24 BTV serotypes (Fig. 2), which is a broader range of detection than reported for the real time PCR assay (15 of 24 prototypic BTV serotypes were detected; Jimenez-Clavero et al., 2006). The primer binding sites of all full length BTV S10 genes that could be retrieved from gene bank and/or ABADRL unpublished data were compared to further understand the specificity of these primers (Table 2). Sequence comparisons suggest that the primer binding sites are highly conserved among all BTV serotypes and topotypes. Of the 82 sequences analyzed, only two BTV isolates (BTV-1 strain 2172 and BTV-12 strain 2016) had minor differences in the nucleotide sequence (Table 2). Such minor differences in the primer binding sites did not elude detection by IR-RT-PCR (see Fig. 4).

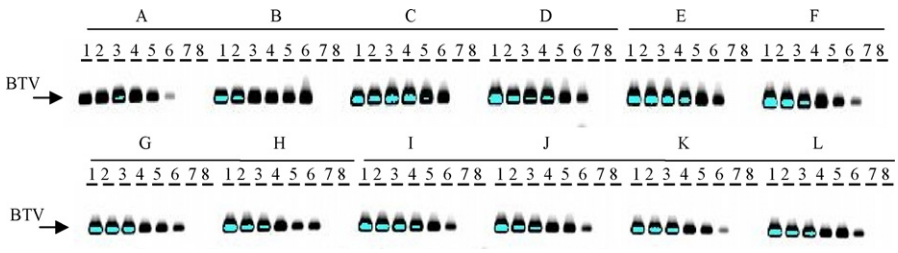

Fig. 3. High-throughput processing of individual *Culicoides* spiked with dilutions of BTV-11, numbers 1–7 (250, 50, 25, 5, 2.5, 0.5, and 0 pfu/reaction, respectively), and 8 is the non-template control. Three dilution series were prepared and assayed as four replicates (set 1, A–D; set 2, E–H; set 3, I–L).

Table 2
Primer binding sites of 82 BTV S10 sequences were compared

Serotype	Strain (**accession number**)	Forward primer binding site (5′–3′)	Reverse primer binding site (5′–3′)
BTV-1	600558*, AUS (**D00253**)	tcgctgccatgctatccg	ctgcattcgcatcgtacg
BTV-2	600557*, 4913/02 (**AY775153**), 6049/02 (**AY775154**), COR2000 (**AF481092**) COR2002 (**AF481093**), OnaB (**L08628**), SA-VAC (**AY775152**), Tunisia (**AF469114**)		
BTV-3	600565*, 2058 (**AY426599**), 2165 (**AY426601**), 2230 (**AY426600**)		
BTV-4	600566*, 2002 (**AY775159**), 2227 (**AY426602**), 320226 (**AY775158**), 322222 (**AY775157**), 10353/03 (**AY775155**), 9034/03 (**AY775156**), IAH-SPA2003/01 (**AJ783910**), IAH-SPA2003/02 (**AJ783911**), SA-VAC (**AY775160**)		
BTV-5	600567*		
BTV-6	600568*, 2187 (**AY426603**)		
BTV-7	600561*		
BTV-8	570600*, 2215 (**AY426604**)		
BTV-9	600571*, 12217/00 (**AY775161**)		
BTV-10	10B80Z (**AF044379**), 10B81U (**AF044381**), 10B81X (**AF044382**), 10B90Z (**AF044384**), 10O90H (**AF044385**), PRO (**AF044372**), US-10O80Z (**AF044380**), US-VAC (**AF044376**)		
BTV-11	11B91P (**AF044383**), 11C81Z (**AF044703**), 11O79X (**AF044386**), 11O81X (**AF044704**), 11B80Z (**AF044702**),PRO (**AF044373**), US-VAC (**AF044377**)		
BTV-12	600604*		
BTV-13	13B80Z (**AF044711**), 13B81K (**AF044712**), 13B89Z (**AF044710**), 13O79Z (**AF044713**), ATCC (**AF044374**), PRO*		
BTV-14	600572*		
BTV-15	600576*		
BTV-16	600577*, 243277 (**AY775163**), 8054/02 (**AY775162**), SA-VAC (**AY775164**)		
BTV-17	283 (**AY426596**), 298 (**AY426597**), 17B80Z (**AF044705**), 17B81Y (**AF044707**), 17B90Z (**AF044708**), 17O79Y (**AF044706**), 17O90Y (**AF044709**), ATCC (**AF044375**), PRO (**L08630**), VAC (**AF044378**)		
BTV-18	600578*		
BTV-19	600579*		
BTV-20	ODV*		
BTV-21	PRO*		
BTV-22	ODV*		
BTV-23	PRO*		
BTV-24	PRO*		
BTV-1	2172 (**AY426598**)	tcgctgccatgctatccg	ctgcattcgcatc**a**tacg
BTV-12	2016 (**AY426595**)	tcgctg**t**catgctatccg	ctgcattcgcatcgtacg

Differences in primer binding sites are indicated in bold underlined letters. Asterisks denote ABADRL, unpublished data.

Furthermore, no cross-reactivity was observed when the BTV IR-RT-PCR was tested with EHDV RNA. EHDV is a closely related orbivirus with high sequence homology to BTV. The sequence similarity among the orbiviruses coupled with the sequence diversity of BTV serotypes complicates the identification of an effective primer set for either real time or nested PCR because of the additional probe or primer binding sites that need to be identified.

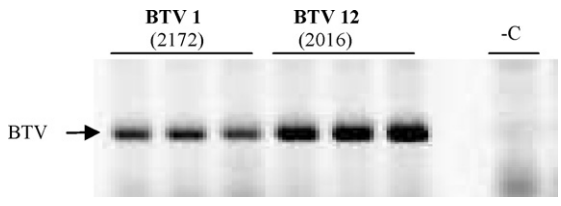

Fig. 4. IR-RT-PCR analysis of BTV-1 2172, and BTV-12 2016, in triplicate. -C designates non-template control. Strain shown in parentheses.

The primers used in this assay were previously described by Akita et al. (1992). The single amplification RT-PCR protocol reported by Akita et al. (1992) targets the S10 gene (NS3 gene product involved with virus egress from insect cells) and has a sensitivity of 100 fg (500 calculated infectious units or 5000 virus particles for BTV-11). The assay reportedly detects 8 BTV serotypes (2, 4, 6, 10, 11, 13, 16, and 17) and neither of the EHDV serotypes (1 and 2) analyzed in the study.

Defined primers were used to illustrate that the IR-RT-PCR method is highly adaptable to preexisting primers with established specificities. The sensitivity of the PCR assay can be increased an estimated 1000-fold (from 500 infectious units as described in Akita et al., 1992, to 0.5 pfu).

BTV RNA is a difficult starting template for RT-PCR because of its double stranded and segmented RNA genome. However, the single amplification RT-PCR was easily converted to a highly sensitive assay utilizing IR dye labeled primers (Akita et al., 1992). The adaptability of the IR-RT-PCR assay allows for a

rapid response in an outbreak situation, and/or the ability to quickly develop and perform sensitive assays for other nucleic acid targets of interest.

For double stranded RNA templates, the denaturation step is critical for primer binding and reverse transcriptase activity. Many BTV PCR methods use toxic chemicals, e.g. methylmercuric hydroxide, to denature the RNA target and achieve greater assay sensitivity (Aradaib et al., 1998; Katz et al., 1993; Parsonson and McColl, 1995; Shad et al., 1997; Wilson, 1999; Wilson and Chase, 1993). The optimization of a typical heat denaturation protocol eliminates the use of toxic chemicals, the production of hazardous waste, and reduces sample manipulation (chemical neutralization is also no longer needed).

The high-throughput method presented here also describes an effective homogenization procedure for individual *Culicoides*. Standard methods for processing individual *Culicoides* for cell culture and PCR assay are labor intensive and often are inconsistent with regard to the degree of maceration. Individual *Culicoides* specimens are difficult to homogenize with mechanical agitators because of the low body mass and the hydrophobic exoskeletons impede standard homogenization beads from impacting the specimens. The homogenization of individual midges has consequently been a manual process taking 2–3 min per sample using motor-driven polypropylene pestles with individual microcentrifuge tubes that can result in increased temperatures of the samples. The homogenization beads used here have not been described previously and can be used in 96 well format.

The One-step Taqman RT-PCR kit was used because of its capacity to amplify low quantities of RNA. Reactions were scaled down 76% from the manufacturer's protocol so that the entire reaction could be loaded within one (15 μl) well in a 100–200 lane gel. The small reaction volume (12 μl) reduces cost, as a 200 reaction kit can assay four-fold the samples. The ROX component of the kit is unnecessary for the reaction; however, it does not diminish assay performance and having a 4 °C stable kit obviates defrosting assay reagents. Only two components (2× Master mix and 40× Multiscribe enzyme) are combined and added to the denatured RNA/primer mix. The reproducibility of the assay is increased because pipetting errors are reduced when only two reagents are combined.

Assay efficiency and throughput capacity can be increased by simultaneously processing two 96 well plates. This allows 192 reactions to be fractionated on a single gel. When running 192 samples on one gel, the agarose concentration is increased from 3% to 3.5% and four (50 well) combs are cast in the gel instead of 2. The gel run time and scan duration remain the same. Throughput can also be increased by pooling *Culicoides* and subsequent analysis.

Multiplexing is possible with IR-RT-PCR method. A second IR dye emitting at ∼700 nm can be used to differentiate targets. The LI-COR Odyssey scanner can simultaneously read two different dyes. The products of BTV IR-RT-PCR can also be visualized on the LI-COR sequencing apparatus (gel and sample preparation modifications: heat denaturation of samples, 5% urea gel, and 50% formamide loading buffer, unreported data). In this case, size discrimination can theoretically be achieved at single nucleotide differences. Additional multiplexing can be accomplished by the addition of a real time PCR primer/probe set to the IR-RT-PCR assay. Initial viral or gene specific information can be accumulated by Taqman real time PCR assay followed by fragmentation of products by gel electrophoresis and IR detection. Neither primers nor probes appear to interfere with either real time or IR-RT-PCR (unpublished).

Single tube multiplex RT-PCR arbovirus detection assays are highly desirable because of the efficiency at which numerous targets can be screened. Ohashi et al. (2004) have developed an assay that simultaneously recognizes four arbovirus serogroups common to Japan. The four virus serogroups include EHDV, BTV, and Palyam (genus *Orbivirus* family Reoviridae); and Simbu serogroup (genus *Orthobunyavirus* family Bunyaviridae). Size discrimination of the amplified gene products allows for serogroup identification to sensitivities comparable to cell culture. Ohashi et al. (2004) report a lower sensitivity limit of 10 $TCID_{50}$ for BTV serotype 20 however the North American BTV-17 strain cannot be detected.

Detection of BTV in experimentally infected *Culicoides* indicates that the IR-RT-PCR method is more sensitive than cell culture procedures. Upon ingestion of a viremic blood meal, BTV must overcome infection barriers for a midge to become competent of transmission (reviewed by Mellor et al., 2000). If the virus cannot transit all barriers, the *Culicoides* may become persistently infected and carry low levels of BTV (Fu et al., 1999). Infectious virus concentrations play a role in detection by cell culture and IR-RT-PCR. When *Culicoides* samples were spiked with known amounts of BTV, and subsequently subjugated to tissue culture analysis, CPE could always be identified in the well containing 25 pfu. At 5 and 2.5 pfu/well, only one of four replicates (from three dilution sets) exhibited CPE, while the IR-RT-PCR assay consistently identified BTV in samples with as little as 0.5 pfu. Many factors can lower virus infectivity in cell cultures. Mertens et al. (1996) has shown that the aggregation of BTV particles in homogenate solutions can reduce infectivity by 10-fold. Furthermore, the state of the virus particle also influences infectivity in both mammalian and insect cell lines. Infectious subviral particles (ISVP) are believed to be formed by the cleavage of outer capsid proteins by digestive enzymes (Mertens et al., 1996). In this state, infectivity of mammalian cell cultures remains the same as disaggregated virus, but increases approximately 100-fold in insect cell lines. BTV core particles (containing dsRNA genome, inner capsid proteins, active RNA polymerase, and capping enzymes) also decrease cell culture infectivity.

In the IR-RT-PCR assay a positive product results from the amplification of specific RNA molecules, therefore, the starting template in this case may be RNA message, genomic RNA from infectious virus, subviral, defective particles, and/or virus aggregates. Virus isolation can only detect live virus, and to differing extents of infectivity, subviral particles and virus aggregates. The increased sensitivity of the IR-RT-PCR assay over cell culture may therefore indicate the presence of target RNA templates and do not necessarily reflect the presence of cell culture infectious virus. In practical terms, the presence of BTV RNA in natural populations of competent *Culicoides* spp. (capable of

transmission or persistently infected) indicates the existence of a reservoir source of BTV in the environment. Steps can then be taken to increase surveillance in these areas, and/or the information may be combined with computer modeling and BTV vector distribution data for risk assessment purposes.

Current OIE recommendations emphasize BTV vector surveillance and the identification of *Culicoides* spp. BTV transmission competent vectors (MacLachlan and Osburn, 2006; OIE, 2004). The IR-RT-PCR assay is suitable for high-throughput surveillance of BTV vectors and transmission competence studies of individual *Culicoides*.

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This article reports the results of research only. Mention of a proprietary product does not constitute an endorsement or a recommendation by the USDA for its use.

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